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Colour and fluorescence emission of *Euchroea auripigmenta* beetle

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ABSTRACT

In nature, many natural organisms display very conspicuous visual appearances. Most of these appearances are due to pigments located within the biological tissues. In addition, fluorescence emission is also known to arise from several organisms. Upon contact with liquids, the colours and fluorescence of some species such as a few from the class Insecta were reported to change reversibly. However, these optical effects are so far not totally elucidated. In this study, the colour and fluorescence properties of *Euchroea auripigmenta* beetle were investigated. This insect exhibits a yellow visual appearance on its head, thorax and elytra when it is illuminated by either visible white light or UV light. After soaking into liquids, both the colour and the fluorescence emission from its integuments are modified. The displayed colour turns from yellow to brown. Using optical, fluorescence and electron microscopy techniques, we morphologically characterised the beetle's integuments. This allowed to observe spike-like protuberances covering the yellow areas of the beetle and from where the yellow visual appearance originates. These protuberances are thought to give rise to further light scattering in addition to the scattering by pigments. Thanks to spectrophotometry, imaging scatterometry and spectrofluorimetry observations, the reflectance and fluorescence properties of this beetle were characterised. Whereas the liquid-induced colour change is attributed to a change in the scattering pattern, the fluorescence emission is most likely due to a chemical influence of the liquids on the two different types of embedded fluorophores.

Keywords: Color, fluorescence, natural photonics, beetle, Coleoptera, biomaterials

1. INTRODUCTION

In the realm of nature, a lot of organisms, including insects, birds, mammals, fishes and plants, exhibit very conspicuous appearances and striking optical effects.[1–5] Their colours are usually related to intra- and interspecific communication, such as for visual recognition, courtship and aposematism, as well as thermoregulation and camouflage. The largest majority of colours in natural organisms is due to pigments such as melanin, ommochromes, pterins and carotenoids.[6] In addition, fluorescence emission was observed from many biological systems including arthropods (e.g., butterflies, beetles and scorpions), fishes or plants.[7–26] These organisms emit visible light when they are illuminated by UV light. This phenomenon is due to fluorophores, such as biopterin, embedded within the biological materials constituting the organisms. Upon contact with liquids, both colour and fluorescence passive changes were also observed in several insect species.[25–35] These changes are general reversible and explained either by the filling of pores present in the insects' tissues, by the swelling of these tissues or by a combination

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of both. In this study, we investigated the case of *Euchroea auripigmenta* (Gory & Percheron, 1835), which is a species from the family Scarabaeidae and the subfamily Cetoniinae (Fig. 1a). This beetle is approximately 2-3 cm long and is found in Madagascar. It displays a yellow colour on its head, thorax and elytra under both visible white and UV incident light (Fig. 1a,b). The name of this species, "auripigmenta", stands indeed for "golden pigment". Upon contact with liquids the colour and fluorescence emission from its tissues are modified (Fig. 1c-f). Its colour turns to brown whereas the visual appearance due to fluorescence emission turns to greenish yellow. Using optical, fluorescence and electron microscopy techniques, we morphologically characterised the beetle's integuments. This allowed to observe spike-like protuberances covering the yellow areas of the beetle and from where the yellow visual appearance originates. These protuberances are thought to give rise to further light scattering in addition to the scattering by the pigments. Thanks to spectrophotometry, imaging scatterometry and spectrofluorimetry observations, the reflectance and fluorescence properties of this beetle were characterised.

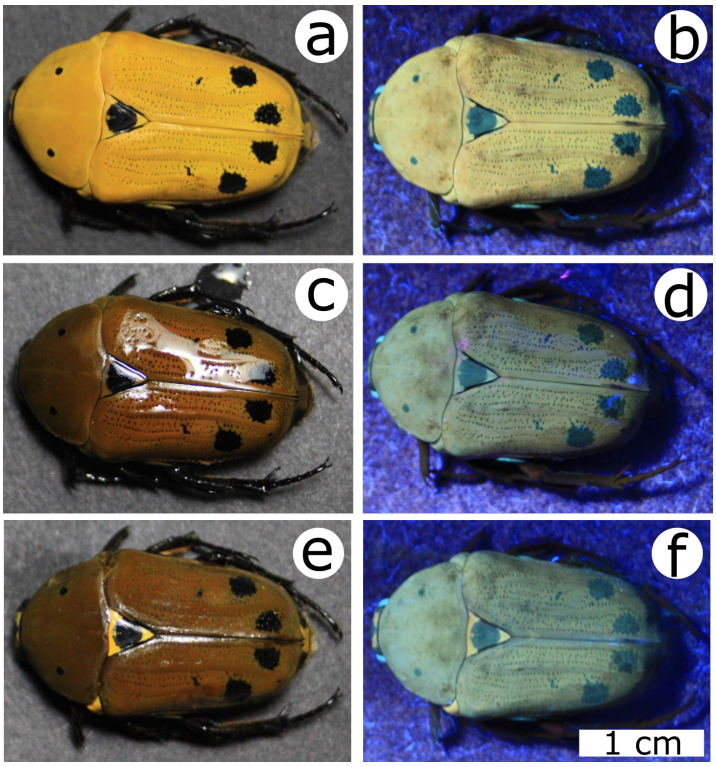

Figure 1. *E. auripigmenta* beetle displays a striking yellow colouration when illuminated by visible white light (a) and UV light (b), giving rise to fluorescence emission, in the latter case. Upon contact with water (c,b) and IPA (e,f), the beetle's colour turns to brown (c,e) whereas the visual appearance due to fluorescence decay turns to greenish yellow (d,f).

2. MATERIALS AND METHODS

2.1 Samples

Samples of dead *E. auripigmenta* beetles were bought from an authorised vendor. No further sample preparation was necessary. Colour and fluorescence changes were performed by soaking elytra detached from the beetle's body into water and isopropanol (IPA) for a sufficient amount of time, i.e., a few tens of minutes, in order to give rise to the observed colour change from yellow to brown. After the measurements, the samples were allowed to dry.

2.2 Morphological characterisation

Samples were photographed using a Canon (Tokyo, Japan) EOS 1000D camera equipped with an objective Canon EF-S 18-55 mm and a Canon EF12 II extension tube, under visible light and UV light from a halogen

lamp and a Labino (Stockholm, Sweden) UV Midlight source, respectively. A black piece of paper was used as background.

Optical microscopy observations were performed using a Zeiss (Oberkochen, Germany) Axioskop 2 MAT mot microscope, a Zeiss AxioCam MRc 5 camera and a Zeiss HAL 100 light source. The elytra of the investigated specimens were detached from the bodies and placed on microscope slides. The flatter areas of the samples were observed. Due to the surface shape of the elytra, an extended depth of focus function was used in order to obtain in-focus microscopic images.

Scanning electron microscopy (SEM) analysis were carried out with a FEI (Hillsboro, Oregon, USA) Nova 600 Nanolab Dual-Beam FIB/SEM. Beetle elytra were cut into pieces of about 5×5 mm^2 and attached to the SEM sample mount by conducting adhesive tape. It was sputter-coated with 25 nm of gold-palladium (AuPd) using a Cressington (Watford, England, UK) sputter coater 208HR.

The elytra were also studied using a JEOL (Tokyo, Japan) JEM-1400 Transmisson Electron Microscope (TEM). The samples were prepared following a standard TEM sample preparation method.[36] 70 nm-thick cross-sections were ultramicrotomed and placed on TEM grids. These cross-sections were also observed by optical microscopy and fluorescence microscopy using an Olympus (Tokyo, Japan) BX61 microscope, an Olympus XC50 camera, a halogen Osram (Munich, Germany) HLX 64625 visible white light source (in reflection mode) and a Lumen Dynamics (Mississauga, Ontario, Canada) X-cite Series 120PCQ UV-lamp (in fluorescence mode).

2.3 Optical and fluorescence characterisation

The reflection factor spectra $R = \frac{I(\lambda)-B(\lambda)}{W(\lambda)-B(\lambda)}$, i.e., the ratios between the intensities I reflected by the beetle elytra and the intensity W reflected by an Ocean Optics (Largo, Florida, USA) WS-1 reflectance standard, with noise corrections B, were measured with an Ocean Optics USB2000 fibre spectrophotometer and an Ocean Optics HPX-2000 xenon lamp. The incident beam formed a 1.5 mm-diameter spot on the sample and the detection area had a diameter equal to ca. 3 mm, thanks to collimating lenses. The integration time of the spectrophotometer was set to 30 ms and each measured spectrum is the result of the average over 10 measurements. Spectra were measured in distoproximal (incidence from the distal end towards the centre of the body), proximodistal (incidence from centre of the body towards the distal end), anteroposterioral (from front to back) and posteroanterioral (from back to front) configurations. Results are reported in this article only in the distoproximal configuration, with the incidence angle θ_i equal to 0°, -15°and -45°with respect to the direction normal to the sample surface. These results are consistent with the other configurations and the other selected incidence angles. From the measured spectra, the displayed colours were quantified in terms of chromaticity coordinates $(x; y)$, assuming the standard illuminant D65 defined by the Commission Internationale de l'Éclairage (CIE) and following a method presented elsewhere.[37,38] They were represented in the CIE 1931 colour space chromaticity diagram.

Imaging scatterometry[39–41] was used in order to observe the far-field spatial distribution pattern of light scattered by the samples. The imaging scatterometer is mainly composed of an ellipsoidal mirror. Small samples are positioned at the first focal point, facing the mirror, thanks to a pipette. Light scattered from the beetles' elytra is reflected by the mirror to the second focal point and is transferred by lenses to a CCD camera, allowing to visualise the scattering pattern. Two incidence configurations were used: a large-angle incidence due to light focused at the second focal point of the ellipsoidal mirror and a narrow-beam incidence due to light collimated on the sample through a small hole in the centre of the mirror. For this study, a small sample was taken from the yellow area of one *E. auripigmenta* beetle's elytron. The acquired scatterograms were corrected for aberrations. A piece of MgO was used as the white standard.

The measurements of the fluorescent characteristics were conducted with an Edinburgh Instruments (Livingston, Scotland, UK) FLSP920 UVvisNIR spectrofluorimeter equipped with a Hamamatsu (Hamamatsu City, Japan) R928P photomultiplier-tube. They were also performed in distoproximal, proximodistal, anteroposterioral and posteroanterioral configurations, with an angle of the excitation beam equal to 45°. Results are reported in this article in the distoproximal configuration but are in agreement with those in the other configurations. The spot size associated with the spectrofluorimeter has a diameter equal to approximately 0.75 mm. The emitted light was detected at a 45° angle on the other side of the direction normal to the sample. Both excitation and emission spectra of dry samples as well as after soaking into water and IPA were measured using a 450 W xenon

lamp as the excitation source. The spectral resolution was equal to 3 nm. The measured emission spectra were fitted by two-term Gaussian functions $A = A_0 + A_1 \exp\left\{-0.5((\lambda - \lambda_{c1})/w_1)^2\right\} + A_2 \exp\left\{-0.5((\lambda - \lambda_{c2})/w_2)^2\right\}$, where A, A_0, A_1 and A_2 are intensities; λ is the wavelength; λ_{c1} and λ_{c2} are the centres of Gaussian peaks; and w_1 and w_2 are the standard deviations. Time-resolved measurements of the intensity emitted by fluorescence from the beetle elytra samples were recorded in dry states and after soaking in water and IPA using a picosecond pulsed EPLED light source, operating at a frequency of 10 MHz with an excitation wavelength equal to 370 nm (i.e., close to the sample excitation peak wavelength in dry state and after soaking in water) and 379 nm (i.e., close to the sample excitation peak wavelength after soaking in IPA). The recorded time-resolved dynamics was fitted by single exponential functions. These numerical fits allowed us to determine the decay time of the fluorescence emission at different excitation wavelengths.

3. RESULTS AND DISCUSSION

E. auripigmenta beetle displays a yellow colour on its head, thorax and elytra (Fig. 1a). Such yellow colourations in insect integuments are usually associated with pigments such as carotenoids and pterins.[6] Black spots of different sizes recover its body. Two large ones on each elytron and one on the scutellum whereas smaller ones are regularly spaced in parallel lines on its cuticle (Fig. 1a and Fig. 2a,b). Under incident UV light, this beetle also display a yellow colouration (Fig. 1b) due to fluorescence emission from the beetle integuments. Interestingly, upon contact with water and liquid IPA, both the beetle's colour and fluorescence emission are reversibly modified (Fig. 1c-f). The colour displayed under visible white light by the head, thorax and elytra changes from yellow to brown. Similarly, the visual appearance under UV incident light is also modified. Upon drying, the visual appearance of the beetle returns to its initial state. After soaking in IPA, the colour of the head and the scutellum does not seem affected by the contact with this liquid (Fig. 1e,f). This allows to observed clearly the changes in the optical response.

SEM observations allow to identify the presence of pits where the small black spots are observed (Fig. 2c,d) and spike-like protuberances or vertical scales on the yellow areas of the cuticle of the elytra (Fig. 2d-f). The pattern formed by these protuberances appears rather disorganised. In addition, TEM analyses indicate the existence of a porous structure under the protuberance layer (Fig. 3a,b).

The spectral reflectance on the yellow area covered by spike-like protuberances was measured by spectrophotometry in the distoproximal configuration for different incidence and detection angles (Fig. 4) and the related chromaticity coordinates were represented in the CIE 1931 chromaticity diagram. All spectra display a low reflection factor below 450 nm, an increase in intensity between 450 nm and 600 nm and a plateau above 600 nm. This gives rise to chromaticity coordinates relatively constant with respect to the incidence and detection angles as they are all located around $(4.5; 4.5)$ (Fig. 4b,d,f), similarly to some tortoise beetles exhibiting golden colourations.[42,43] Typically, the plateau corresponds to the highest intensities in configurations close to a specular configuration. These intensities uniformly decreases when the detection angle moves away from the specular configuration. A local maximum of the intensity of the plateau is observed when the configuration is close to a backscattering configuration. In addition, with an incidence angle $\theta_i = -45°$, the reflection factor spectra become slightly desaturated with increasing detection angles θ_r, leading to chromaticity coordinates a bit more desaturated (Fig. 4e,f). This optical behaviour was captured in a single figure thanks to imaging scatterometery (Fig. 5) with both large- and narrow-angle incidences. The exhibited yellow colouration is relatively homogeneous in hue and appears on a relatively large solid angle (Fig. 5b), unlike the golden visual appearances of the mentioned tortoise beetles[42,43] that are metallic (i.e., very directional light reflection). If this homogeneous scattering pattern can be associated with pigmentary colours, the relative disorder observed in the spike-like protuberances (Fig. 2d-f) most likely fosters multidirectional light scattering from the beetle's tissues.

Passive colour changes in insects' integuments are usually associated with the filling of the pores in their cuticles.[25–28,30–35] Chitin, which is the main material composing the insects' integuments is known to be microporous (i.e., containing pores the diameters of which are smaller than 2 nm).[25,44–49] In addition, mesopores (i.e., with pore diameters between 2 and 50 nm) and macropores (i.e., with pore diameters larger than 50 nm) can also be observed in insects' cuticles.[1–5,26–28] In the case of *E. auripigmenta* beetle, this colour change can be explained by the change of refractive index surrounding the yellow pigments, following the filling of the cuticle pores by the liquid. Light scattering by the pigments is modified, on a way similar to a wet white t-shirt, fostering

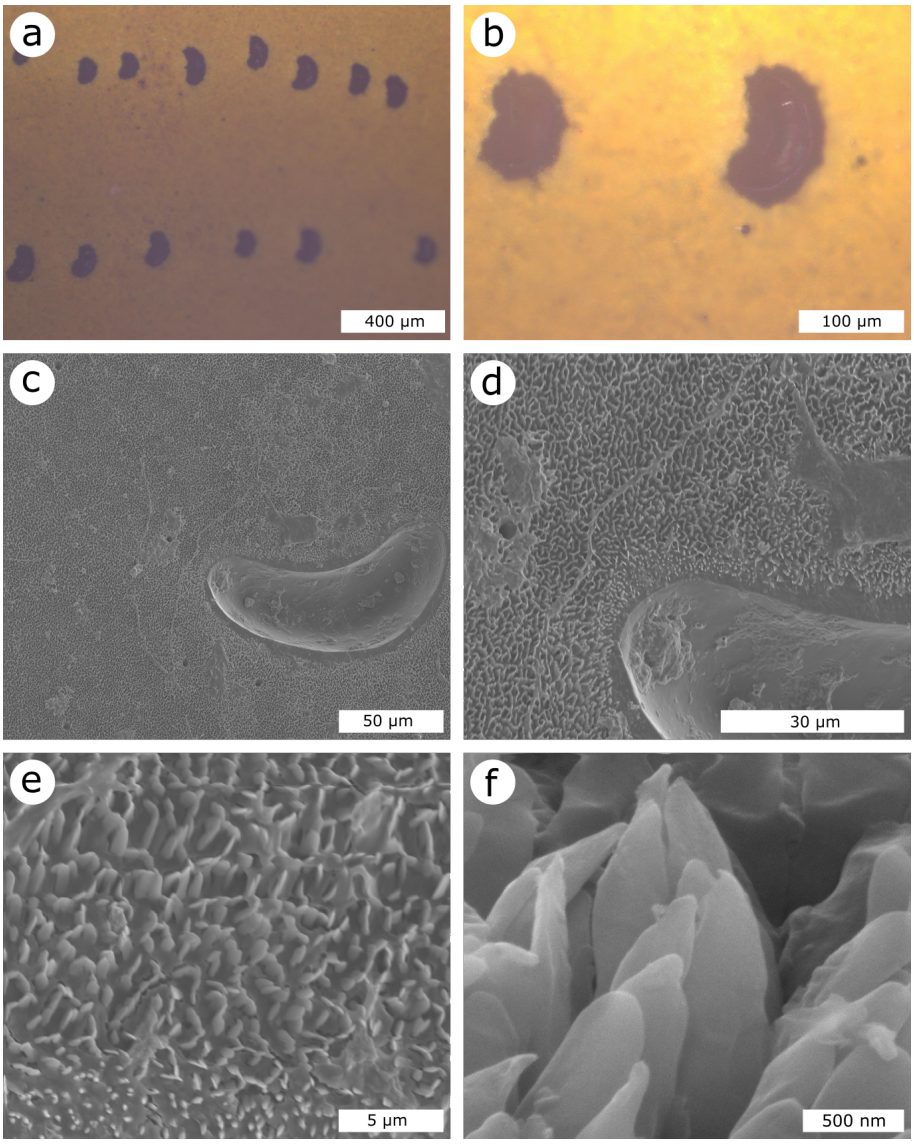

Figure 2. The elytra of *E. auripigmenta* exhibit a black-spotted yellow colouration in the dry state. These bean-shaped black spots are quite regularly displayed in lines on the cuticle, as observed by optical microscopy (a,b). SEM observations reveal that the black spots correspond to pits in the beetle's cuticle (c,d) whereas the yellow area is covered by spike-like protuberances (e,f).

forward scattering and unveiling an underlying layer containing brown pigments such as melanin. After soaking in IPA, the head and the scutellum of *E. auripigmenta* were observed not to change. This may be explained by the non-infiltration of IPA into the related tissues, possibly due to different chemical properties of these parts of the beetle's body.

Under a fluorescence microscope (Fig. 3e), the spike-like protuberances observed by TEM (Fig. 3c) and optical microscopy (Fig. 3d) emit light by fluorescence decay. The fluorescent characteristics of *E. auripigmenta*'s integuments were measured by spectrofluorimetry (Fig. 6). In the dry state, the excitation spectrum peaks at 367 nm whereas the emission spectrum is broad with a maximum located at 544 nm and a shoulder around 460 nm. The corresponding positions of the two fitted Gaussian functions ($R^2 = 0.993$) are found at 549.25±0.25 nm and 457.61±0.57 nm with standard deviations equal to 74.53 nm and 20.98 nm, respectively. Upon contact with

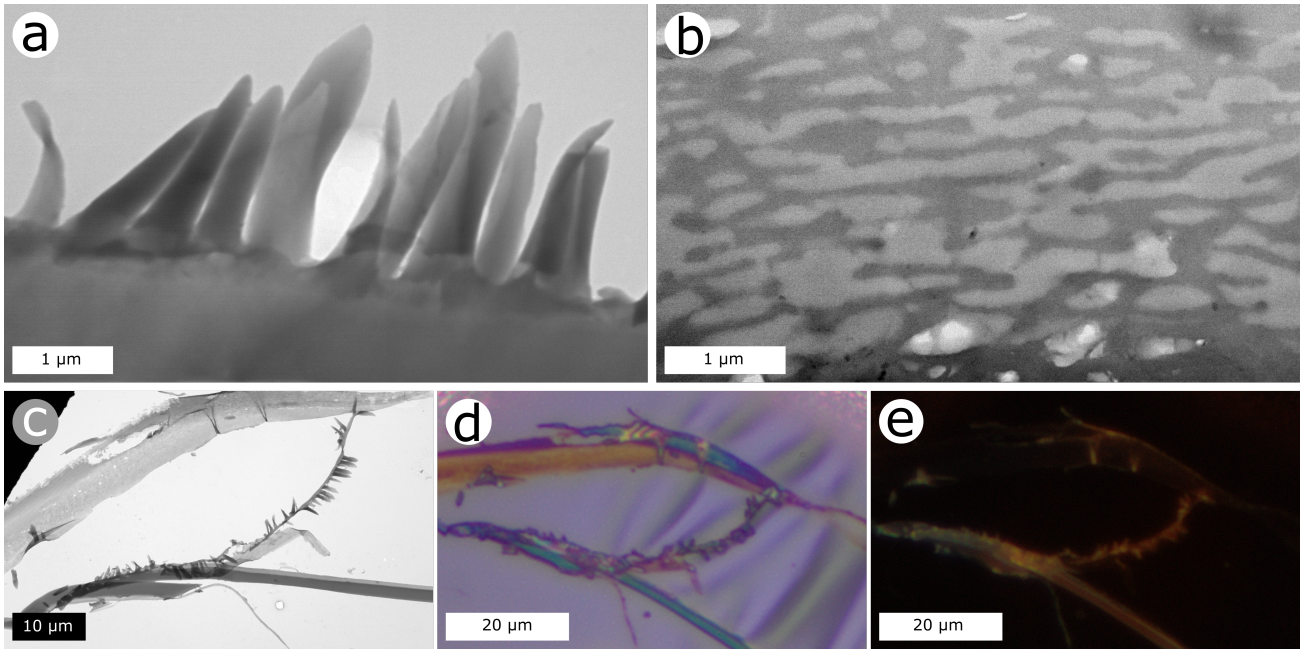

Figure 3. TEM analysis showed the presence of spike-like protuberances covering the cuticle of *E. auripigmenta*'s elytra (a). Underneath this protuberance layer, the beetle's integuments are porous (b). Observation of the same cross-section by TEM (c), optical microscopy (d) and fluorescence microscopy (e) demonstrate the emission of light by fluorescence decay from the spike-like protuberances.

liquids, these spectra are modified. The excitation spectra corresponding to water and IPA exhibit a maximum at 377 nm and 376 nm, respectively. The modification of the emission spectrum is even more striking. Upon contact with water, the peak measured at 544 nm in the dry state relatively decreases in intensity so that it matches the intensity of the shoulder, leading to a broad flat spectrum between ca. 460 nm and ca. 560 nm (with its centre at 507 nm). The positions of the two Gaussian functions ($R^2 = 0.991$) are 461.86±0.51 nm and 545.87±1.60 nm with standard deviations equal to 26.96±1.21 nm and 72.68±1.60 nm, respectively. In the case of IPA, the emission spectrum displays a peak at 459 nm and a shoulder at about 550 nm, where the emission spectrum of the beetle in the dry state exhibits a shoulder and a peak, respectively. The centres of the related Gaussian functions ($R^2 = 0.991$) are 455.13±0.39 nm and 535.12±2.17 nm with standard deviations equal to 27.46±1.00 nm and 73.54±1.91 nm, respectively. These measurements confirm the observed greenish yellow visual appearance. Whatever the liquid, the Gaussian peaks are located very close to the positions of the corresponding peaks in the dry state. In addition, their standard deviations are also very similar to the ones of the corresponding peaks before soaking in liquids. Only their relative intensities are modified. The chemistry of *E. auripigmenta*'s fluorophores has not been yet identified. However, the change in these fluorescent characteristics upon contact with liquids is most likely due to a chemical effect: the presence of the liquid influences the fluorophores embedded within the beetle's tissues. This combination of a peak and a shoulder within the same emission spectra and the different ways by which these components of the spectra are affected after soaking in water and IPA suggests the presence of at least two kinds of fluorophores. The variation in peak intensities may be due to a difference in light scattering at the surface of the sample arising from the presence of liquid. In addition, the measurements could not be performed exactly at the same location on the sample, possibly leading to such differences in intensities. Time-resolved measurements of the emitted intensity allowed to determine the decay time of the fluorescence emission in the dry state as well as after soaking the samples into water and IPA (Table 1). The excitation wavelength was selected at 370 nm, i.e., around the maximum of the excitation spectra (Fig. 6). The emission wavelengths were chosen to be at both edges of the emission bands, i.e., 400 nm and 625 nm, as well as around the emission peaks, i.e., 550 nm (for the dry state and water) and 462 nm (in the case of IPA). In all three cases, the decay time strongly depends on the emission wavelength (Table 1), suggesting also

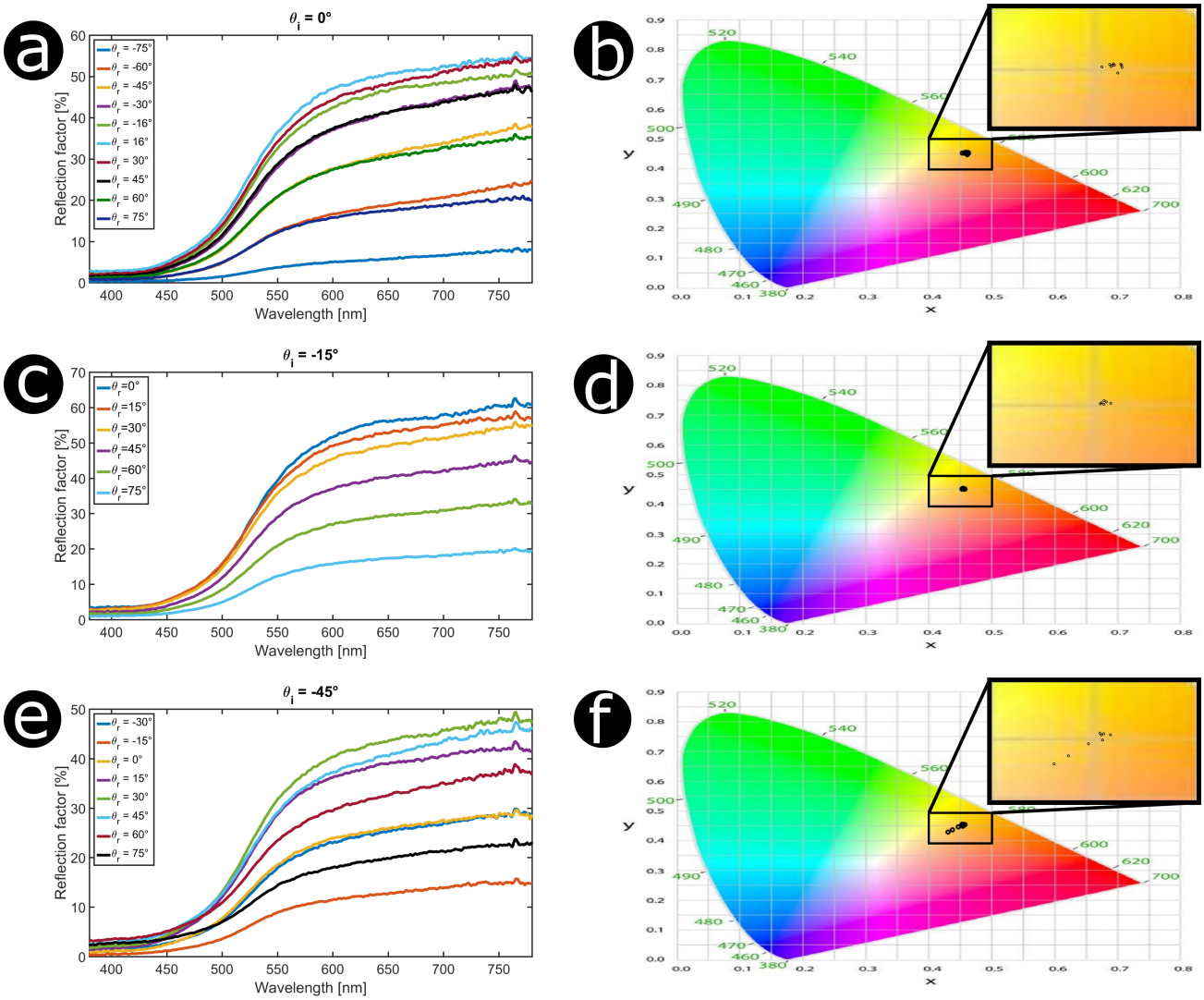

Figure 4. Spectra of the reflection factor measured from *E. auripigmenta* beetle's elytra by spectrophotometry in a distoproximal configuration for different incidence angles θ_i and detection angles θ_r (a,c,e) all exhibit a low intensity below ca. 450 nm, an increase in intensity between 450 nm and 600 nm and a plateau above 600 nm. Therefore they correspond to similar chromaticity coordinates located around $(4.5; 4.5)$.

different fluorophores are present in the beetle's cuticle. In addition, the contact with liquids clearly influences the decay time at these wavelengths on different ways (Table 1). Such a result also suggests that the change in fluorescence emission is due to a chemical effect.

4. CONCLUSION

The head, thorax and elytra of *E. auripigmenta* beetle exhibit a yellow visual appearance under both white visible and UV incident light. The former is scattered by yellow pigments such as carotenoids and pterins in the beetle's cuticle, giving rise to the observed yellow colouration. The latter gives rise to light emission by fluorophores naturally embedded within the integuments of the beetle. Observations using different microscopy techniques showed that this yellow visual appearance originates from an area covered by spike-like protuberances, which possibly give rise to an additional light scattering. This yellow colouration is quite homogeneous with respect to the direction of light incidence and observation, as showed by spectrophotometry and scatterometry

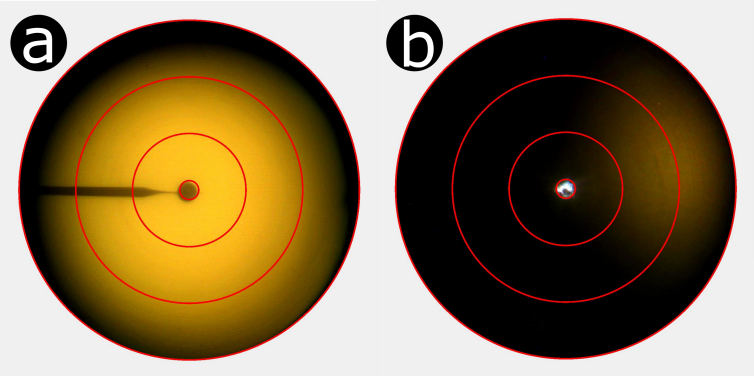

Figure 5. Light scattered by the elytra of *E. auripigmenta* exhibits a homogeneous hue over a large range of detection angles, with both with both large- (a) and narrow-angle (b) incidences, as observed by imaging scatterometery.

Table 1. Decay times τ and related standard errors (s.e.) of the fluorophores embedded in *E. auripigmenta* beetle's cuticle in dry state as well as after soaking in water and in IPA. The excitation wavelength was selected at 370 nm, i.e., around the maximum of the excitation spectra. The emission wavelengths were chosen at both edges of the emission bands, i.e., 400 nm and 625 nm, as well as around the emission peaks, i.e., 550 nm (for the dry state and water) and 462 nm (in the case of IPA). The incident light formed a 45°angle with the direction normal to the sample surface. The emitted light was detected at a 45°angle on the other side of the normal direction, in a distoproximal configuration.

λ_{em} (nm)	$\tau_{Dry}\pm$ s.e. (ns)	$\tau_{Water}\pm$ s.e. (ns)	$\tau_{IPA}\pm$ s.e. (ns)
400	0.759 ± 0.026	1.179 ± 0.064	1.036 ± 0.042
550 (Dry and Water) 462 (IPA)	1.026 ± 0.047	1.557 ± 0.098	1.017 ± 0.059
625	1.027 ± 0.073	0.609 ± 0.027	1.212 ± 0.116

measurements. Our experiments revealed that the yellow colour and fluorescence emission of this beetle's are modified upon contact with water and IPA. The colour change can be explained by a modification of the light scattering, following the filling of the cuticle material pores with liquid, leading to a change in the refractive index surrounding the pigments. Spectrofluorimetry measurements indicated that probably at least two different types of fluorophores are embedded in the cuticle. In addition, the change in fluorescence emission was attributed to the influence of the liquid on the fluorophores.

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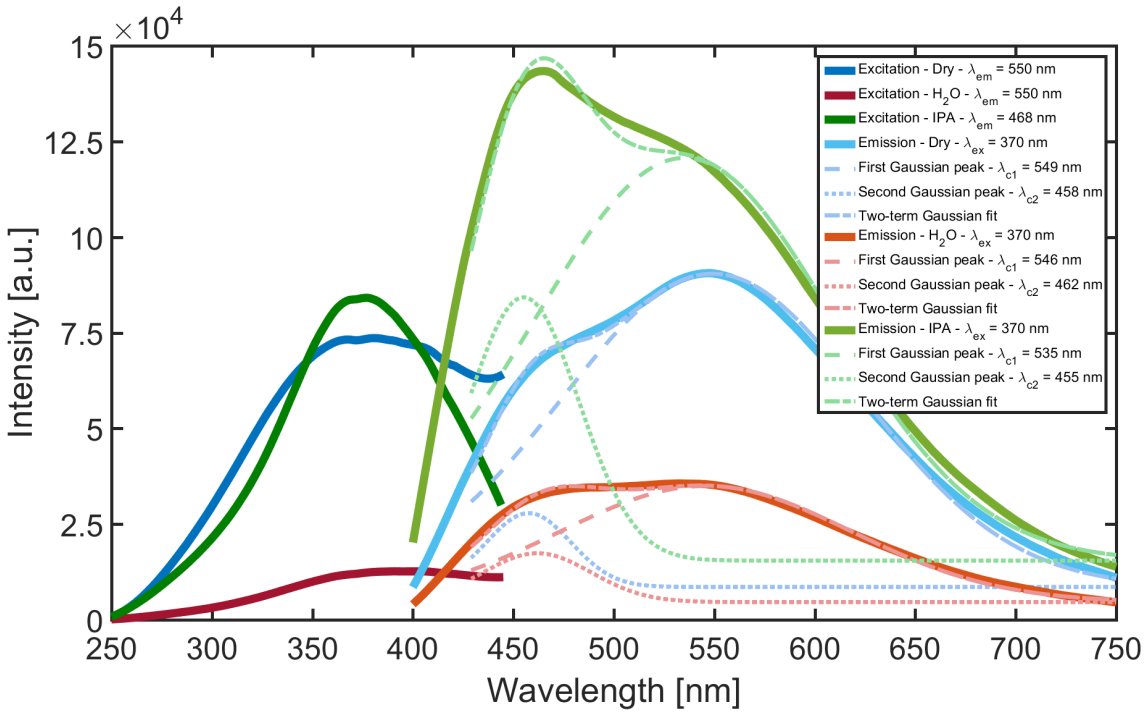

Figure 6. In the dry state, the excitation spectrum measured from the elytra of *E. auripigmenta* beetle peaks at 367 nm whereas the emission spectrum is broad with a maximum located at 544 nm and a shoulder around 460 nm. The corresponding positions of the two fitted Gaussian functions ($R^2 = 0.993$) are found at 549.25±0.25 nm and 457.61±0.57 nm with standard deviations equal to 74.53 nm and 20.98 nm, respectively. The excitation spectra after soaking in water and IPA exhibit a maximum at 377 nm and 376 nm, respectively. The modification of the emission spectrum is even more striking. Upon contact with water, the peak measured at 544 nm in the dry state relatively decreases in intensity so that it matches the intensity of the shoulder, leading to a broad flat spectrum between ca. 460 nm and ca. 560 nm (with its centre at 507 nm). The positions of the two Gaussian functions ($R^2 = 0.991$) are 461.86±0.51 nm and 545.87±1.60 nm with standard deviations equal to 26.96±1.21 nm and 72.68±1.60 nm, respectively. In the case of IPA, the emission spectrum displays a peak at 459 nm and a shoulder at about 550 nm, where the emission spectrum of the beetle in the dry state exhibits a shoulder and a peak, respectively. The centres of the related Gaussian functions ($R^2 = 0.991$) are 455.13±0.39 nm and 535.12±2.17 nm with standard deviations equal to 27.46±1.00 nm and 73.54±1.91 nm, respectively. Dashed lines correspond to the first Gaussian peak intensities $A = A_0 + A_1 \exp\left\{-0.5((\lambda - \lambda_{c1})/w_1)^2\right\}$, dotted lines correspond to the second Gaussian peak intensities $A = A_0 + A_2 \exp\left\{-0.5((\lambda - \lambda_{c2})/w_2)^2\right\}$ and dash-dotted lines correspond to two-term Gaussian fits $A = A_0 + A_1 \exp\left\{-0.5((\lambda - \lambda_{c1})/w_1)^2\right\} + A_2 \exp\left\{-0.5((\lambda - \lambda_{c2})/w_2)^2\right\}$.

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